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(54) ANTITHROMBOTIC AGENT AND ANTI-VON WILLEBRAND FACTOR MONOCLONAL ANTIBODIES

(57) A monoclonal antibody, which has reactivity with human von Willebrand factor, which has action to inhibit RIPA (ristocetin-induced platelet aggregation), BIPA (botrocetin-induced platelet aggregation), and SIPA (shear stress-induced platelet aggregation) of human platelet, and which does not express bleeding action in an medicinally effective dose to exhibit anti-thrombotic action, is used as an active ingredient of an antithrombotic agent.

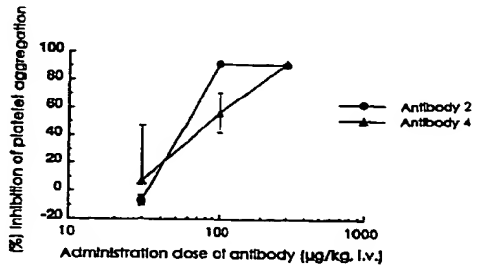

FIG. 21

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state. The vascular diameter is small, and the bloodstream has a large velocity in arteriosclerosis lesions and small arteries. Therefore, a high shear stress occurs in such regions due to the interaction between vessel wall and blood. In such a situation, vWF in blood is activated, and its tertiary structure is changed. As a result, vWF plays a crucial role in thrombus formation. Namely, the following process is known. Firstly, vWF existing on subendothelium binds to GPIb on platelet membrane, and thus platelets adhere to vessel wall. Secondly, vWF existing in blood plasma cross-links glycoprotein IIb/IIIa on platelet membrane, and thus the platelet aggregation reaction is allowed to proceed. Consequently, thrombus formation finally occurs.

It is generally known that an antibiotic ristocetin or a snake venom botrocetin allows vWF to cause a change in tertiary structure in vitro, equivalent to the change under a high shear stress. Namely, in the presence of ristocetin or botrocetin, vWF acquires the binding ability to GPIb. Methods for measuring the physiological activity of vWF in vitro by utilizing the foregoing characteristic include ristocetin-induced platelet aggregation (hereinafter abbreviated as "RIPA") and botrocetin-induced platelet aggregation (hereinafter referred to as "BIPA"), as well as a method for measuring binding of vWF to GPIb in the presence of ristocetin or botrocetin. The foregoing methods are widely utilized. Owing to the progress of experimental techniques, an apparatus has been also developed, in which SIPA is measured in vitro by actually applying a shear stress. It is considered that an identical domain on vWF involved in the binding to GPIb in any of the reactions.

Several antibodies against vWF, which inhibit the activity of vWF in vitro, have been hitherto obtained. However, many of them are inferior in reaction specificity, and almost all of them do not inhibit the botrocetin-dependent reaction, even though they inhibit the ristocetin-dependent reaction. As described above, it is considered that the GPIb-binding site on vWF induced by ristocetin is homologous to that induced by botrocetin. Therefore, the foregoing antibodies possibly recognize the binding site on vWF for ristocetin or botrocetin. Strictly speaking, it is possible to say that they do not inhibit the physiological activity of vWF, and hence they have low reaction specificities. In such circumstances, it has been reported that two antibodies, i.e., NMC-4 produced by Fujimura et al. (J. Nara Med. Assoc., vol. 36, p. 662, 1985) and RFF-VIIIRAG:1 produced by Tuddenham et al., inhibit in vitro the reaction depending on both of ristocetin and botrocetin (Blood, vol. 177, No. 1, p. 113, 1992).

It has been reported that epitopes for the two antibodies exist in the GPIb-binding site of the vWF molecule, and they are located between 449th and 728th amino acid residues of an amino acid sequence of the vWF molecule. Further, binding of iodine-labeled NMC-4 to vWF is partially inhibited by RFF-VIIIRAG:1. According to this fact, it is considered that the both epitopes are located at positions considerably close to one another. Moreover, RFF-VIIRAG:1 inhibits BIPA only partially, while NMC-4 completely inhibits BIPA. For this reason, studies have been diligently made in the scientific field of vWF by using NMC-4, and certain results have been obtained. Among animals other than human, NMC-4 has its reactivity only with rat vWF.

When a monoclonal antibody against human vWF is prepared in order to obtain information on the GPIb-binding site of human vWF, or in order to use the monoclonal antibody as a preventive agent and a therapeutic agent against diseases relevant to vWF, it is considered to be desirable to prepare the monoclonal antibody as one having high specificity to human vWF.

On the other hand, when a new medicine is developed in an ordinary manner, it is unallowable to perform any test with human without previously performing a test with animals. When a test is performed in relation to physiological activities of vWF and anti-vWF monoclonal antibodies in vivo, it is necessary to use a monoclonal antibody which makes it possible to perform a test with animals, i.e., a monoclonal antibody simultaneously having reactivity with vWF of an animal other than human. By the way, GPIIb/IIIa antagonists, which strongly suppresses human platelet aggregation by the aid of fibrinogen, are not effective on rat (Thrombosis and Haemostasis, vol. 70, p. 531, 1993). Further, rat does not cause ristocetin-induced aggregation. According to these facts, it is generally considered that the mechanism of thrombus formation greatly differs between rat and human. Therefore, it is almost meaningless to evaluate the antithrombotic action of any anti-vWF antibody by using rat. On the contrary, in the case of guinea pig, platelet aggregation is suppressed by GPIIb/IIIa antagonists. Further, ristocetin-induced aggregation is also induced in the same manner as human. Accordingly, it is considered that guinea pig is most suitable as an animal thrombus model for in vivo experiments when the antithrombotic action is evaluated.

According to the foregoing viewpoints, any of a monoclonal antibody having reactivity with only human vWF, and a monoclonal antibody having reactivity with both human vWF and guinea pig vWF is useful. However, such anti-human vWF monoclonal antibodies are not known.

Further, an anti-human vWF monoclonal antibody, which has been confirmed to have antithrombotic action in vivo, is not known.

Disclosure of the Invention

The present invention has been made taking the foregoing viewpoints into consideration, an object of which is to provide monoclonal antibodies against human von Willebrand factor, especially a monoclonal antibody having reactivity with only human vWF, and a monoclonal antibody having reactivity with human vWF as well as guinea pig vWF, which do not express bleeding action in an medicinally effective dose sufficient to

monoclonal antibody. The first monoclonal antibody of the present invention is clearly different in epitope from NMC-4 described above not only in that it reacts with animal vWF but also in that it does not inhibit binding of NMC-4 to vWF at all (see Examples described later on). The fact that the monoclonal antibody of the present invention has strongly suppressed thrombus formation without involving the bleeding tendency in an in vivo thrombosis model strongly suggest the possibility that the monoclonal antibody of the present invention can be also utilized as an ideal therapeutic agent for thrombotic diseases. The monoclonal antibody of the present invention is not only novel but also industrially applicable.

Namely, the first monoclonal antibody of the present invention is not only useful to specify the GPIb-binding site of vWF. But the first monoclonal antibody of the present invention is also expected to be used as means for analyzing distribution and existing forms of vWF in vivo, and researching the cause of vWD (von Willebrand disease), and to be utilized as a preventive agent and a therapeutic agent effective on thrombotic diseases. Further, the first monoclonal antibody of the present invention can be preferably used for in vivo experiments based on the use of guinea pig when the antithrombotic action is evaluated.

A second embodiment of the monoclonal antibody of the present invention (hereinafter referred to as "second monoclonal antibody") is a monoclonal antibody having the following properties:

(A) the monoclonal antibody has reaction specificity to human von Willebrand factor;
(B) the monoclonal antibody inhibits RIPA (ristocetin-induced platelet aggregation), BIPA (botrocetin-induced platelet aggregation), and SIPA (shear stress-induced platelet aggregation) of human platelet; and
(C) the monoclonal antibody does not react with von Willebrand factors of rat, guinea pig, and rabbit.

Namely, the second monoclonal antibody of the present invention is reactive with human vWF, and it has high affinity thereto. Further, the second monoclonal antibody strongly inhibits any of RIPA, BIPA, and SIPA in vitro. Besides, the second monoclonal antibody does not react with any of vWF's of rat, guinea pig, and rabbit. In view of these points, the second monoclonal antibody has specificity much higher than that of NMC-4.

No monoclonal antibody having the properties as described above has been also hitherto known. The second monoclonal antibody of the present invention is a novel monoclonal antibody. The second monoclonal antibody is clearly different in epitope from NMC-4 described above not only in that it does not react with rat vWF but also in that it does not inhibit binding of NMC-4 to vWF at all (see Examples described later on). According to the fact that the monoclonal antibody of the present invention does not react with vWF's of those other than human, for example, vWF of rat, it is assumed that the monoclonal antibody of the present invention recognizes a special antigenic determinant specific to human, the antigenic determinant having been not conserved during the process of evolution. This fact is considered to support the high specificity of the monoclonal antibody of the present invention. The monoclonal antibody of the present invention is not only novel but also industrially applicable.

The second monoclonal antibody specifically and strongly inhibits binding between human vWF and GPIb on platelet membrane. Accordingly, the second monoclonal antibody can be utilized as means for specifying the GPIb-binding site of human vWF, analyzing distribution and existing forms of human vWF in vivo, and researching the cause of vWD (von Willebrand disease), in the same manner as the first monoclonal antibody. No in vivo thrombus formation-suppressing experiment has been performed based on the use of animal, because the second monoclonal antibody does not react with vWF's of animals other than human. However, as demonstrated in Examples described later on, an epitope for the second monoclonal antibody to recognize vWF is located in the vicinity of an epitope recognized by the first monoclonal antibody. Accordingly, the second monoclonal antibody highly possibly recognizes the same epitope as that recognized by the first monoclonal antibody. Therefore, it is assumed that the second monoclonal antibody has an effect equivalent to that of the first monoclonal antibody in vivo. The second monoclonal antibody is expected to be utilized as a preventive agent and a therapeutic agent effective on thrombotic diseases.

The first and second monoclonal antibodies also have action to inhibit shear stress-induced platelet adhesion (hereinafter referred to as "SIPAd") of human platelet. SIPAd also relates to thrombus formation in a pathological state. According to an experiment based on the use of normal human blood, it has been confirmed that the first and second monoclonal antibodies inhibit SIPAd in a dose-dependent manner. Such inhibition has not been observed for GIIb/IIIa antagonists which are expected to be used as antithrombotic agents at present.

A third embodiment of the monoclonal antibody of the present invention is a monoclonal antibody which has reactivity with human vWF, and which has action to inhibit binding between the first or second monoclonal antibody and vWF factor when the third monoclonal antibody is allowed to co-exist with the first or second monoclonal antibody. As demonstrated in Examples described later on, one of the first and second monoclonal antibodies mutually inhibits binding of the other to vWF, using epitopes located closely near to one another or using an identical epitope. Further, the first monoclonal antibody has strongly suppressed thrombus formation without accompanying the hemorrhagic tendency in an in vivo thrombus model. According to these facts, the properties possessed by the first and

pig, rat, and rabbit, or a monoclonal antibody that inhibits BIPA or RIPA of platelet of an animal as described above, in accordance with an enzyme immunoassay method such as an ELISA (Enzyme-Linked Immunosorbent Assay) method. The hybridoma, which produces the second monoclonal antibody of the present invention, is obtained by selecting a hybridoma which produces a monoclonal antibody that does not exhibit reactivity with vWF of an animal such as rabbit other than human.

After confirmation of the fact that the hybridoma for producing the objective monoclonal antibody is contained in a culture, the culture is transferred to HT medium having the same composition as that of HAT medium except that aminopterin is removed from HAT medium. The hybridoma is further cultured to perform cloning in accordance with, for example, a limiting dilution method.

Thus hybridomas AJvW-1, AJvW-2, AJvW-3, and AJvW-4 have been obtained as demonstrated in Examples described later on. All of them have been deposited on August 24, 1994 in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology of Ministry of International Trade and Industry (postal code: 305, 1-3 Higashi-Icchome, Tsukuba-shi, Ibaraki-ken, Japan) under deposition numbers of FERM P-14486, FERM P-14487, FERM P-14488, and FERM P-14489 respectively in this order, which have been transferred to international deposition based on the Budapest Treaty on September 29, 1995, and deposited under deposition numbers of FERM BP-5247, FERM BP-5248, FERM BP-5249, and FERM BP-5250 respectively in this order. Among the hybridomas, AJvW-2 and AJvW-4 produce the first monoclonal antibody, and AJvW-1 and AJvW-3 produce the second monoclonal antibody.

As demonstrated in Examples described later on, the monoclonal antibodies produced by AJvW-1 and AJvW-3 belong to the subclass IgG2a isotype, the monoclonal antibody produced by AJvW-2 belongs to the subclass IgG1, and the monoclonal antibody produced by AJvW-4 belongs to the subclass IgG2b. NMC-4 belongs to IgG1 as having been hitherto reported.

The monoclonal antibody of the present invention is obtained by culturing, in an appropriate medium or in mouse ascitic fluid, the hybridoma obtained as described above or a variant selected by cloning the hybridoma in accordance with the limiting dilution method, for example, a variant of the hybridoma having high antibody productivity. Alternatively, the monoclonal antibody of the present invention is also obtained by isolating a gene concerning antibody production from the obtained hybridoma, incorporating the gene into an expression vector, introducing an obtained vector into a microorganism such as Escherichia coli, and cultivating an obtained antibody-producing microorganism. The hybridoma includes AJvW-1, AJvW-2, AJvW-3, AJvW-4 described above, and variants thereof.

The medium for culturing the hybridoma includes, for example, a medium based on DMEM medium and further containing fetal bovine serum, L-glutamine, glucose, sodium pyruvate, 2-mercaptoethanol, and an antibiotic (for example, penicillin G, streptomycin, and gentamicin). The hybridoma of the present invention is usually cultured in the medium at 37 °C for 2 to 4 days with a gas phase comprising 5 % carbon dioxide and 95 % air. Alternatively, the hybridoma is cultured for about 10 to 15 days in an abdominal cavity of Balb/c mouse pretreated with 2,6,10,14-tetramethylpentadecane (for example, Pristane (trade name) produced by Sigma). Thus the monoclonal antibody is produced in an amount capable of being subjected to purification.

The monoclonal antibody thus produced can be separated and purified in accordance with an ordinary method adopted for isolation and purification of proteins from culture supernatant or ascitic fluid. Such a method includes, for example, centrifugation, dialysis, salting out based on the use of ammonium sulfate, and column chromatography based on the use of, for example, DEAE-cellulose, hydroxyapatite, protein-A agarose, and protein-G agarose.

(3) Antithrombotic agent of the present invention

The antithrombotic agent of the present invention contains, as an active ingredient, the monoclonal antibody which has reactivity with human von Willebrand factor, which has action to inhibit RIPA (ristocetin-induced platelet aggregation), BIPA (botrocetin-induced platelet aggregation), and SIPA (shear stress-induced platelet aggregation) of human platelet, and which does not express bleeding action in an medicinally effective dose to exhibit antithrombotic action. Such a monoclonal antibody specifically includes the first monoclonal antibody and the second monoclonal antibody of the present invention. As described above, it is expected that the monoclonal antibody, which has action to inhibit binding between the first and second monoclonal antibodies and vWF factor when the monoclonal antibody is allowed to co-exist with the first and second monoclonal antibodies, also has the same action as those of the first and second monoclonal antibodies, and it is used as an active ingredient of the antithrombotic agent of the present invention.

When the monoclonal antibody originating from mouse is applied as an antithrombotic agent to human, it is desirable that the monoclonal antibody is modified into one of the human type, because of problems of antigenicity and half-life in blood. Variable regions of the antibody can be converted into those of the human type without losing the reaction specificity in accordance with methods described by Jones et al. (Nature, vol. 321, p. 522, 1986) and Queen et al. (Proc. Natl. Acad. Sci. USA, vol. 86, p. 10029, 1989). Recently, the repertoire cloning method described by Winter et al. and Lerner et al. is also available (J. Mol. Biol., vol. 222, p. 581, 1991; Proc. Natl. Acad. Sci. USA, vol. 88, p. 2432, 1991).

Fragments $F(ab')_2$, Fab', and Fab, which can be

Fig. 25 shows an effect of Antibody 2 on occlusion time in a guinea pig A-V shunt model.

Fig. 26 shows an effect of Antibody 2 on bleeding time in a guinea pig bleeding time model.

Fig. 27 shows an effect of Antibody 4 on the bleeding time in a guinea pig bleeding time model.

Best Mode for Carrying Out the Invention

The present invention will be more specifically explained below making reference to Examples. However, the present invention is not limited to Examples described below.

Examples

(1) Preparation of monoclonal antibodies

(1) Immunosensitization and cell fusion

Purified human vWF was mixed with an equal amount of an adjuvant (MPL+TDM EMULSION; trade name of RIBI), and an obtained mixture was subcutaneously administrated to Balb/c male mice (8 weeks old upon the start of immunization) in an amount corresponding to an amount of vWF of 100 μg per 1 mouse (priming immunization). After 21 days, immunization was performed by subcutaneous administration in the same manner as described above (booster immunization). After 19 days or 30 days from the booster, the mice were administrated through their tail veins with 200 μl of a preparation obtained by diluting human vWF with PBS (phosphate-buffered physiological saline, produced by Nissui) to have a concentration of 250 μg/ml (final immunization).

Spleens were excised from the mice after 3 days from the final immunization, and they were separated into single cells. Subsequently, the spleen cells were washed with DMEM medium. On the other hand, mouse myeloma cells Sp2/0-Ag14 in the logarithmic growth phase were collected, and they were washed with DMEM medium. The spleen cells and the mouse myeloma cells were sufficiently mixed in a plastic tube in a ratio of numbers of the cells of 10:1, followed by addition of 50 % (w/v) polyethylene glycol (produced by Boehringer Mannheim, average molecular weight: 4000) to perform cell fusion at 37 °C for 7 minutes.

A supernatant solution was removed by means of centrifugation, and a residue is added with HAT medium (DMEM medium containing 10 % fetal bovine serum added with hypoxanthine, aminopterin, and thymidine). The residue was suspended so that the concentration of the spleen cells was 5 x 10^6 cells/ml. This cell suspension was dispensed and poured into 96-well plastic plates so that one well contained 100 μl of the suspension, followed by cultivation at 37 °C in 5 % carbon dioxide. HAT medium was supplemented in an amount of 50 μl/well on 2nd and 5th days. After that, half volume of the medium was exchanged every 3 or 4 days in conformity with proliferation of hybridomas.

(2) Screening and cloning of hybridomas

Hybridomas, which produced the monoclonal antibody of the present invention, were screened by using, as an index, the inhibitory activity of the monoclonal antibody on the physiological activity possessed by vWF. A part of the medium in each of the wells after completion of proliferation of hybridomas was sampled, for which the inhibitory activities on RIPA and BIPA were measured. Hybridoma clones, which strongly inhibited the both reactions, were selected.

Hybridomas, which produced monoclonal antibodies exhibiting reactivity with vWF's of guinea pig, rabbit, and rat, were selected from the selected clones. The obtained hybridomas were transferred to HT medium which was the same as HAT medium except that aminopterin was removed from HAT medium, and they were further cultured. Cloning was performed twice in accordance with the limiting dilution method. Thus stable hybridomas were obtained. Finally obtained two hybridomas were designated as AJvW-2 and AJvW-4.

On the other hand, hybridomas, which produced monoclonal antibodies exhibiting no reactivity with vWF's of guinea pig, rabbit, and rat, were selected from the clones which strongly inhibited the reactions of RIPA and BIPA described above. The obtained hybridomas were transferred to HT medium which was the same as HAT medium except that aminopterin was removed from HAT medium, and they were further cultured. Cloning was performed twice in accordance with the limiting dilution method. Thus stable hybridomas were obtained. Finally obtained two hybridomas were designated as AJvW-1 and AJvW-3.

AJvW-2 and AJvW-4 produced the first monoclonal antibody of the present invention, and AJvW-1 and AJvW-3 produced the second monoclonal antibody of the present invention.

(2) Production and purification of monoclonal antibodies

(1) Production of monoclonal antibodies

2,6,10,14-Tetramethylpentadecane (trade name: Pristane, produced by Sigma, 0.5 ml) was intraperitoneally injected into Balb/c female mice which were 6 to 8 weeks old from the birth. After 10 to 20 days, cells of AJvW-1, AJvW-2, AJvW-3, or AJvW-4 (1 x 10^6 to 10^7 cells) were suspended in PBS, and they were intraperitoneally inoculated into the mice. After 7 to 10 days, the mice were sacrificed and subjected to an abdominal operation, from which produced ascitic fluid was collected. The ascitic fluid was centrifuged to remove insoluble matters, and a supernatant was recovered and stored at -20 °C.

obtained as described above to give a final concentration of 5 μg/ml, and platelet aggregation was measured in accordance with the same method as that described in the foregoing item (1). Results are shown in Fig. 4 (Antibodies 2, 4) and Fig. 5 (Antibodies 1, 3). Any of Antibody 1, Antibody 2, Antibody 3, Antibody 4, and NMC-4 inhibited BIPA in a dose-dependent manner. IC_{50} values were 0.8 μg/ml for Antibody 1, 2.0 μg/ml for Antibody 2, 1.0 μg/ml for Antibody 3, 5.6 μg/ml for Antibody 4, and 2.0 μg/ml for NMC-4.

Guinea pig PRP was prepared in the same manner as described above, to which botrocetin was added to give a final concentration of 2 μg/ml, and measurement was performed in the same manner as described above. Antibody 1 (final concentration: 80 μg/ml), Antibody 3 (final concentration: 80 μg/ml), and NMC-4 (final concentration: 27 μg/ml) did not inhibit BIPA at all, while Antibody 2 and Antibody 4 inhibited BIPA in a dose-dependent manner (Fig. 6). IC_{50} values were 3.1 μg/ml for Antibody 2 and 3.5 μg/ml for Antibody 4.

PRP was prepared by centrifuging citrated blood of rat at 1300 rpm for 10 minutes. Botrocetin was added to PRP (5×10^8 platelets/ml) to give a final concentration of 0.08 μg/ml, and measurement was performed in the same manner as described above. Antibody 1 (final concentration: 80 μg/ml) and Antibody 3 (final concentration: 80 μg/ml) did not inhibit BIPA of rat at all, while Antibody 2, Antibody 4, and NMC-4 inhibited BIPA in a dose-dependent manner (Fig. 7). Values of IC_{50} were 1.2 μg/ml for Antibody 2, 5.0 μg/ml for Antibody 4, and 2.2 μg/ml for NMC-4.

PRP was prepared by centrifuging citrated blood of rabbit at 1200 rpm for 10 minutes. Botrocetin was added to PRP (3×10^8 platelets/ml) to give a final concentration of 0.075 μg/ml, and measurement was performed in the same manner as described above. Results are shown in Fig. 8 (Antibodies 2, 4) and Fig. 9 (Antibodies 1, 3). Antibody 1 (final concentration: 80 μg/ml), Antibody 3 (final concentration: 80 μg/ml), and NMC-4 (final concentration: 27 μg/ml) did not inhibit rabbit BIPA at all, while Antibody 2 and Antibody 4 inhibited BIPA in a dose-dependent manner. IC_{50} values were 5.0 μg/ml for Antibody 2 and 1.8 μg/ml for Antibody 4.

(3) Inhibitory activities on SIPA

Human PRP (2.5×10^8 platelets/ml, 360 μl) was reacted at room temperature for 10 minutes with the monoclonal antibody (40 μl) at respective concentrations. After that, platelet aggregation induced by shear stress was measured by using an apparatus for measuring cell function (produced by Toray). Namely, the reaction mixture was applied with constant shear of 6 dyne/cm^2 during a period of 0 to 15 seconds, low-shear gradient of 6 → 12 dyne/cm^2 during a period of 15 to 105 seconds, high-shear gradient of 12 → 108 dyne/cm^2 during a period of 105 to 225 seconds, and constant shear of 108 dyne/cm^2 during a period up to 350 seconds. The extent of platelet aggregation activity was represented by change in optical transmittance. The inhibitory activity on platelet aggregation was determined by using, as a control, the maximal aggregation obtained by adding DMEM or the buffer for dissolving the sample.

Results are shown in Fig. 10 (Antibodies 2, 4) and Fig. 11 (Antibodies 1, 3). Any of Antibody 1, Antibody 2, Antibody 3, Antibody 4, and NMC-4 inhibited SIPA of human in a dose-dependent manner. IC_{50} values were 0.7 μg/ml for Antibody 1, 1.1 μg/ml for Antibody 2, 0.9 μg/ml for Antibody 3, 1.5 μg/ml for Antibody 4, and 1.5 μg/ml for NMC-4.

〈5〉 Affinity of monoclonal antibodies of the present invention to human vWF

(1) ^{125}I-labeling for human vWF

Iodogen (produced by Pierce, 1 mg/ml) and a dichloromethane solution (1 ml) were added to a polypropylene tube from which the solvent was removed by using nitrogen stream. A solution of human vWF (0.43 mg/ml) was poured into the polypropylene tube to which a solution of Na^{125}I (15.9 MBq, 8.6 μl) was added to perform a reaction at room temperature for 2 minutes. After the reaction, the reaction solution was applied to a PD10 column (produced by Pharmacia) having been previously blocked with a TBS solution (Tris-buffered saline) containing 2 ml of 10 % BSA (bovine serum albumin) and washed with 100 ml of TBS. Elution was performed with TBS. The eluted solution was fractionated into fractions each having a volume of 500 μl. An aliquot (2 μl) of each of the eluted fractions was measured for its radioactivity by using a γ-counter (counting time: 1 minute). Fractions having high counted values were collected, and a combined fraction (1 ml) was designated as a solution of ^{125}I-labeled human vWF (^{125}I-vWF) (0.3 mg/ml human vWF, 220630 cpm/μl).

(2) Preparation of immobilized platelets

Human PRP collected in the same manner as described in the foregoing item 〈4〉 (1) was added and mixed with an equal volume of 2 % paraformaldehyde solution, followed by being left to stand at 4 °C overnight. On the next day, immobilized platelets were recovered and washed three times with PBS by means of centrifugation operation. After that, the immobilized platelets were resuspended in PBS having a volume equal to that for PRP upon the collection, and an obtained suspension was used as an immobilized platelet suspension.

(3) Action of monoclonal antibodies of the present invention on platelet-binding property of vWF

The immobilized platelet suspension, a solution of the antibody at various concentrations, and a ristocetin solution or a botrocetin solution were dispensed and

and Antibody 4 inhibited RIPA in a dose-dependent manner. ED_{50} values (values of 50 % inhibition on platelet aggregation) were 70 μg/kg for Antibody 2 and 90 μg/kg for Antibody 4. In BIPA, Antibody 4 had a ED_{50} value of 55 μg/kg. The strong actions of Antibody 2 and Antibody 4 for inhibiting RIPA and BIPA were continuously observed up to 6 hours from the administration, and disappeared after 48 hours.

Separately from the foregoing test, hematological parameters concerning whole blood collected with citric acid after 5 minutes from the administration of the antibody were measured by using an automated hematology analyser (Sysmex E-2000, produced by Toa Medical Electronics). Antibody 2 or Antibody 4 was administrated in an administration dose of 100 μg/kg or 300 μg/kg. In any case, no significant variation was observed in total platelet count, total red blood cell count, total white blood cell count, hemoglobin concentration, hematocrit value, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red blood cell distribution width, platelet distribution width, mean platelet volume, white blood cell large cell ratio, and platelet hematocrit value.

Blood plasma was separated by means of centrifugation operation from blood obtained 5 minutes after the administration of the antibody, in the same manner as described above. The activated partial thromboplastin time, the prothrombin time, and the fibrinogen concentration were measured for the blood plasma by using a coagulation parameter-measuring apparatus (Sysmex CA-3000, produced by Toa Medical Electronics). As a result, no significant variation was observed in the respective parameters even when Antibody 2 or Antibody 4 was administrated in an administration dose of 1000 μg/kg.

(8) Preventive effects on thrombus formation of monoclonal antibodies of the present invention in guinea pig (in vivo)

1) Evaluation of preventive effects on thrombus formation in photochemically induced thrombus model in guinea pig carotid artery (PIT model)

Occlusive thrombus was allowed to be formed in carotid artery of guinea pig in accordance with a method of Nakajima et al. (Thrombosis Research, vol. 67, p. 435, 1992) so that the time to thrombus formation was measured with or without administration of Antibody 2 or Antibody 4.

Carotid artery of guinea pig was exposed and exfoliated under urethane anesthesia, to which a probe of pulse Doppler blood flowmeter was installed. Antibody 2, Antibody 4, or physiological saline was administrated in an amount of 30, 100, or 300 μg/kg through a cannula attached to the carotid artery. After 5 minutes, a photosensitizing substance, i.e., rose bengal was administrated in an amount of 10 mg/kg through the same cannula. Simultaneously, blood vessel located upstream from the probe-installed site (located on a side of the heart) was irradiated with exciting green light at 540 nm by using a thrombus-producing light source (produced by Hamamatsu Photonics) to measure the time (occlusion time) to occlusion of the blood vessel and bloodstream stop due to thrombus formation.

Results are shown in Figs. 23 and 24. Antibody 2 and Antibody 4 significantly prolonged the occlusion time in an administration dose of not less than 100 μg/kg. Statistical processing was performed by using Mann-Whiteny U test. In Figs. 23 and 24, a symbol * indicates $p < 0.05$, and a symbol ** indicates $p < 0.01$.

(2) Evaluation of preventive effect on thrombus formation based on A-V shunt model

A polyethylene tube was inserted into left jugular vein of guinea pig under urethane anesthesia, through which Antibody 2 or physiological saline was administrated in a dose of 30, 100, or 300 μg/kg. After 5 minutes, an opposite side of the tube was inserted into right jugular vein to form a shunt, and the bloodstream was opened again. The time to bloodstream stop (occlusion time) was measured by using a pulse Doppler blood flowmeter.

Results are shown in Fig. 25. Antibody 2 significantly prolonged the occlusion time in an administration dose of not less than 100 μg/kg. Statistical processing was performed by using Mann-Whiteny U test. In Fig. 25, a symbol * indicates $p < 0.05$, and a symbol ** indicates $p < 0.01$.

(3) Evaluation of prolongation of bleeding time

Antibody 2, Antibody 4, or physiological saline was intravenously administrated to guinea pig in a dose of 100 or 300 μg/kg under pentobarbital anesthesia. After 5 minutes, planta artery was incised over a length of 5 mm. The presence or absence of bleeding from the wound was confirmed every 15 seconds by using, as an index, a bloodstain adhered to filter paper. The time required from the incision to the stop of bleeding was measured.

Results are shown in Figs. 26 and 27. Antibody 2 and Antibody 4 prolonged the bleeding time in a dose of 1000 μg/kg. Antibody 2 and Antibody 4 did not affect the bleeding time at all in an administration dose of 300 μg/kg at which they exhibited the occlusion time-prolonging effect in the PIT model and the A-V shunt model described above. Statistical processing was performed by using Mann-Whiteny U test. In Figs. 26 and 27, a symbol * indicates $p < 0.05$, and a symbol ** indicates $p < 0.01$.

According to the experimental results described above, it has been demonstrated that both Antibody 2 and Antibody 4 exhibit the strong inhibitory action on thrombus formation without expressing the hemorrhagic tendency which would be otherwise cause clinical prob-

brand factor.

14. The hybridoma according to claim 13, which is AJvW-1, AJvW-3, or a variant of any of them.

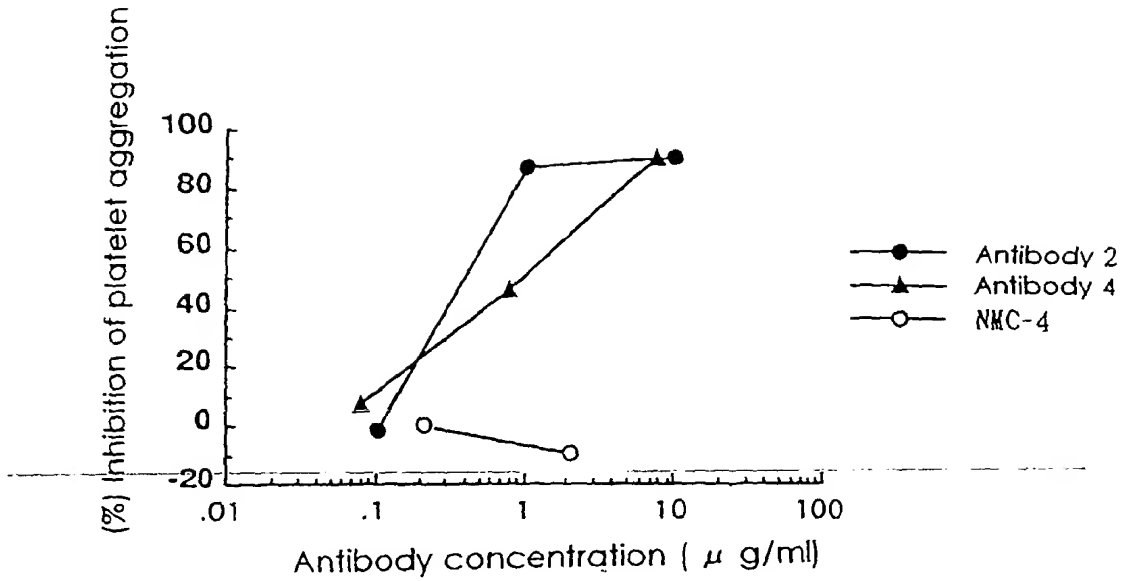
(%) Inhibition of platelet aggregation
100
80
60
40
20
0
-20
.01
.1
1
10
100
Antibody concentration (μ g/ml)
Antibody 2
Antibody 4
NMC-4

FIG. 3

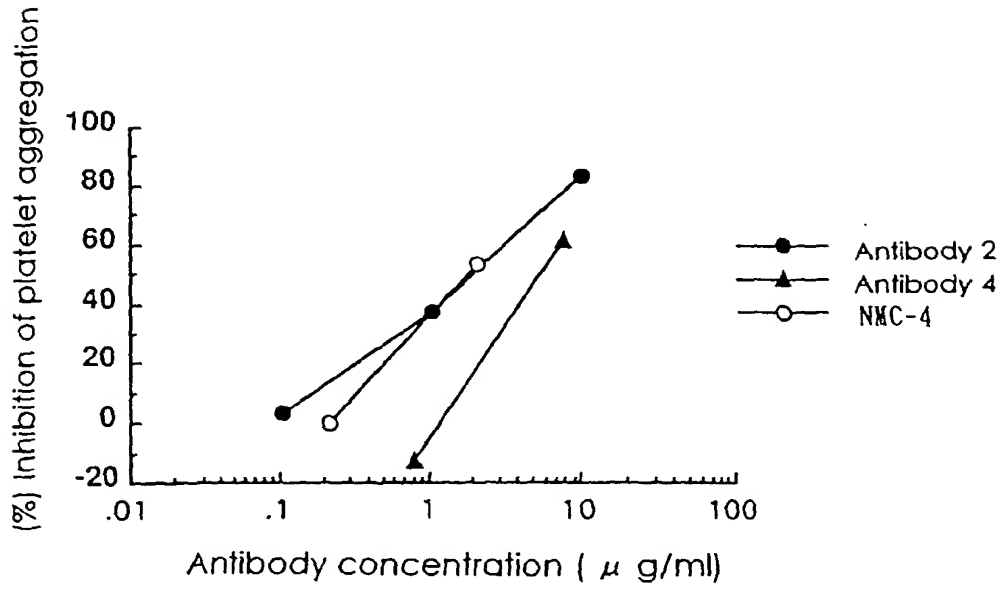
(%) Inhibition of platelet aggregation
100
80
60
40
20
0
-20
.01
.1
1
10
100
Antibody concentration (μ g/ml)
Antibody 2
Antibody 4
NMC-4

FIG. 4

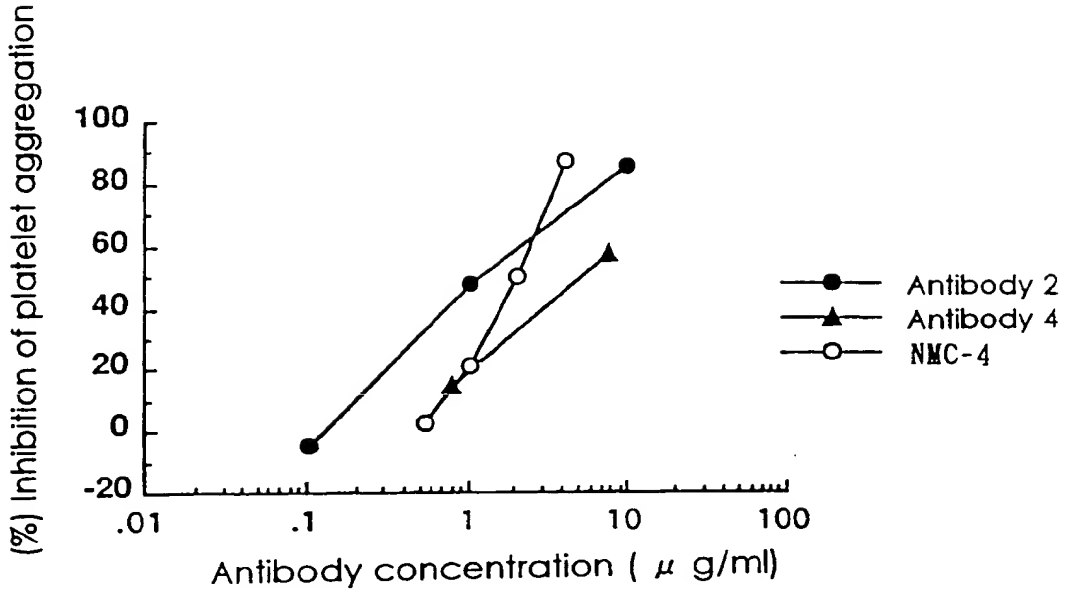
(%) Inhibition of platelet aggregation
100
80
60
40
20
0
-20
.01
.1
1
10
100
Antibody concentration (μ g/ml)
Antibody 2
Antibody 4
NMC-4

FIG. 7

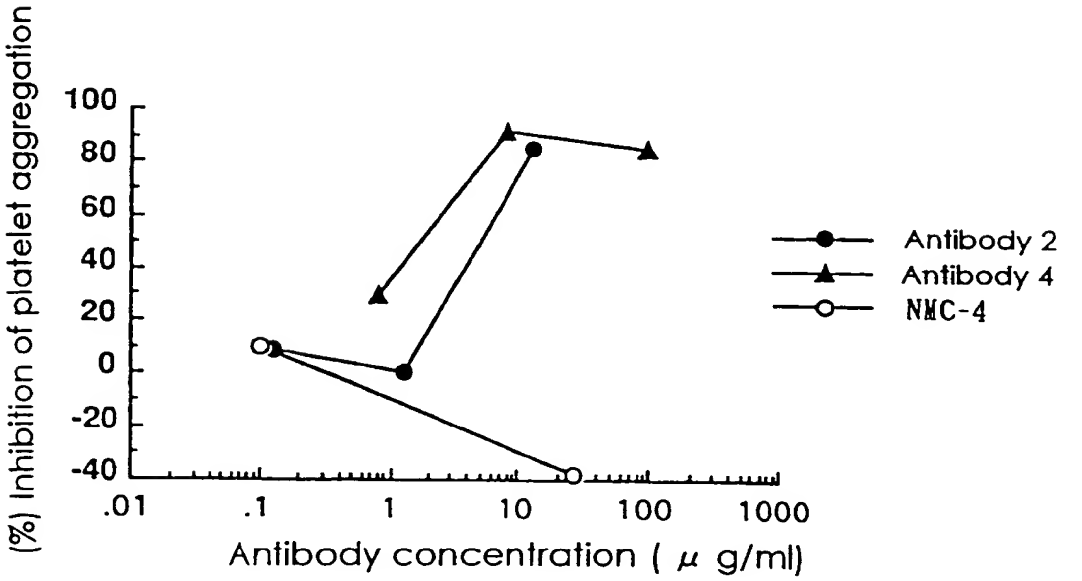
(%) Inhibition of platelet aggregation
100
80
60
40
20
0
-20
-40
.01
.1
1
10
100
1000
Antibody concentration (μ g/ml)
Antibody 2
Antibody 4
NMC-4

FIG. 8

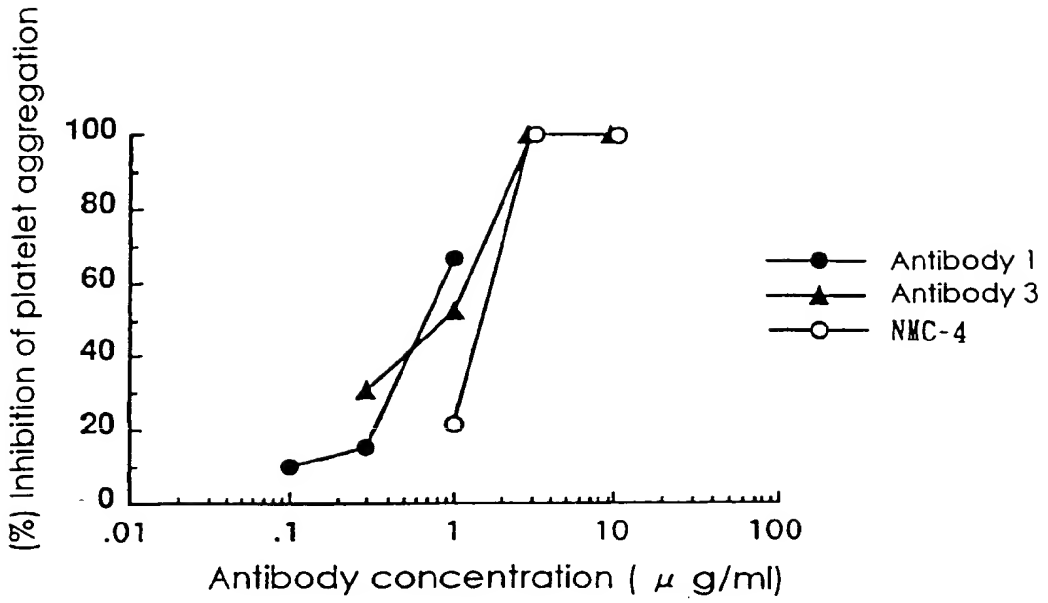
(%) Inhibition of platelet aggregation
100
80
60
40
20
0
.01
.1
1
10
100
Antibody concentration (μ g/ml)
Antibody 1
Antibody 3
NMC-4

FIG. 11

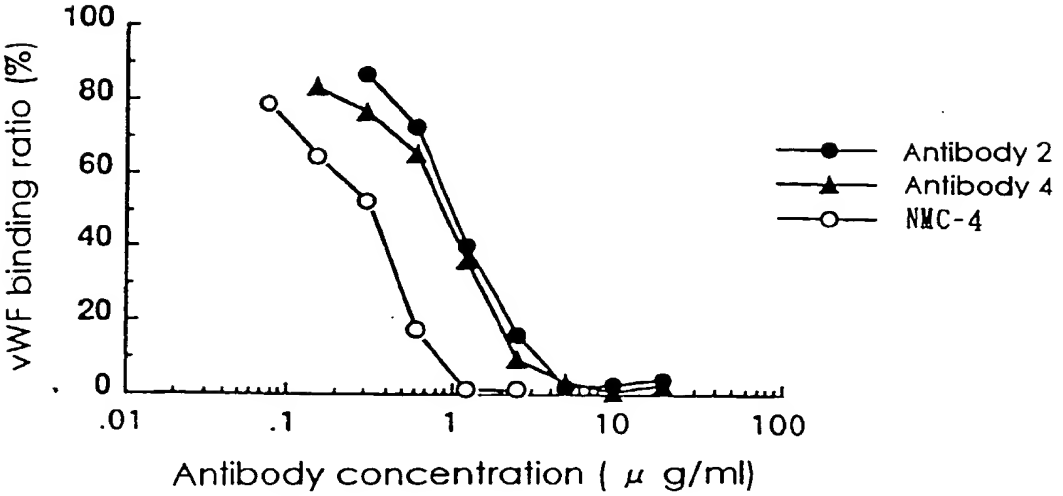
vWF binding ratio (%)
100
80
60
40
20
0
.01
.1
1
10
100
Antibody concentration (μ g/ml)
Antibody 2
Antibody 4
NMC-4

FIG. 12

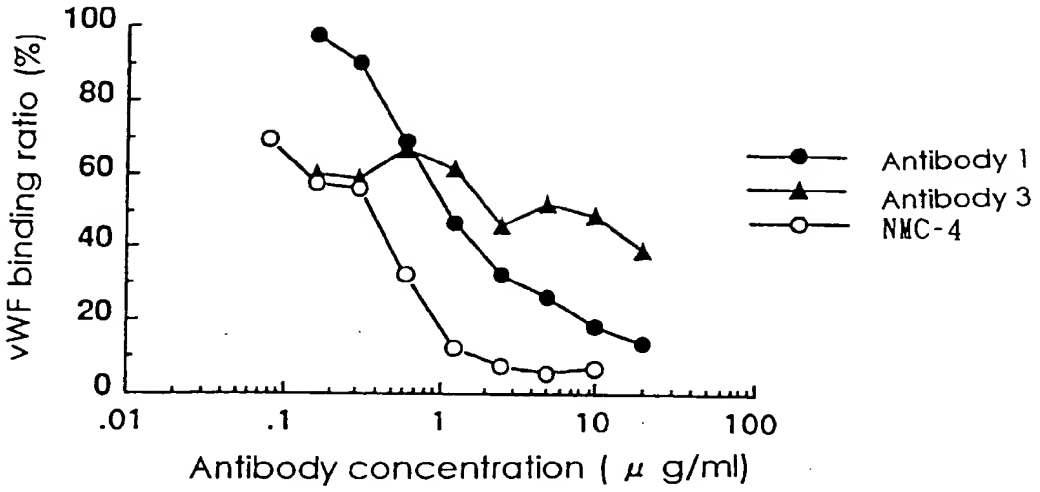

FIG. 15

Effects of Respective Antibodies on Binding of Biotinylated AJvW-1 to Immobilized vWF

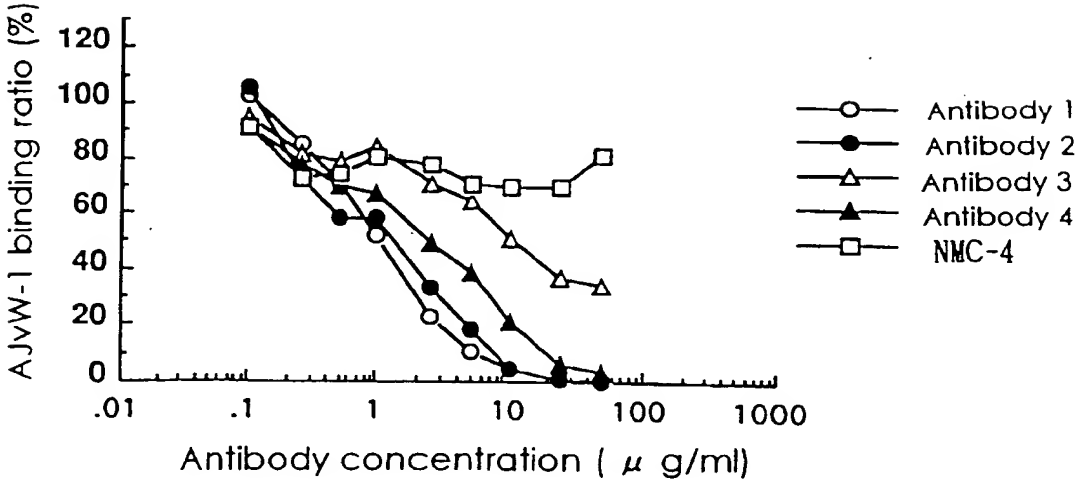

FIG. 16

Effects of Respective Antibodies on Binding of Biotinylated AJvW-4 to Immobilized vWF

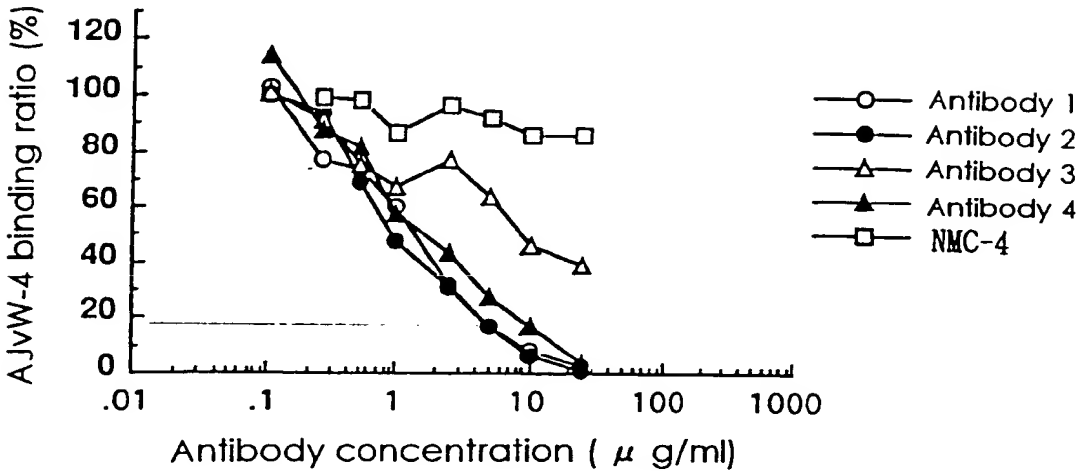

FIG. 19

Effects of Respective Antibodies on Binding of Biotinylated NMC-4 to Immobilized vWF

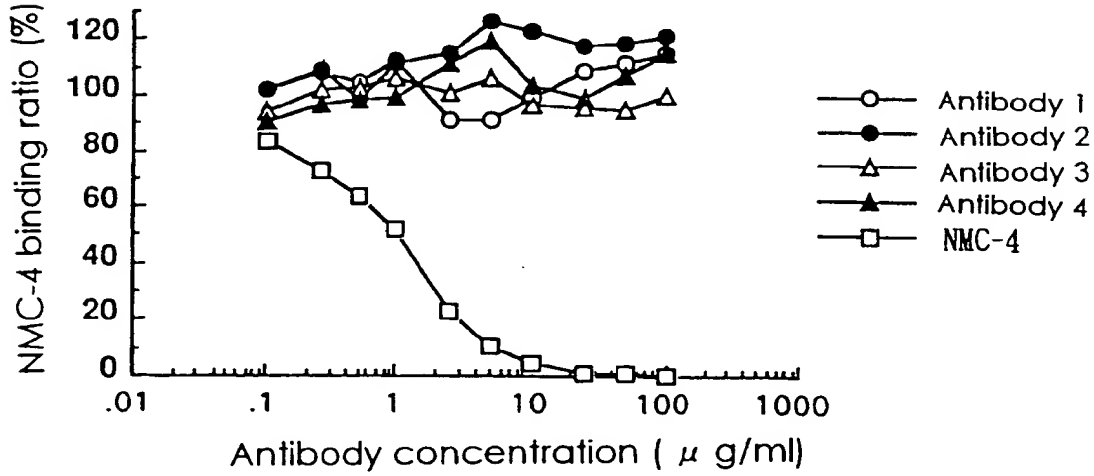

FIG. 20

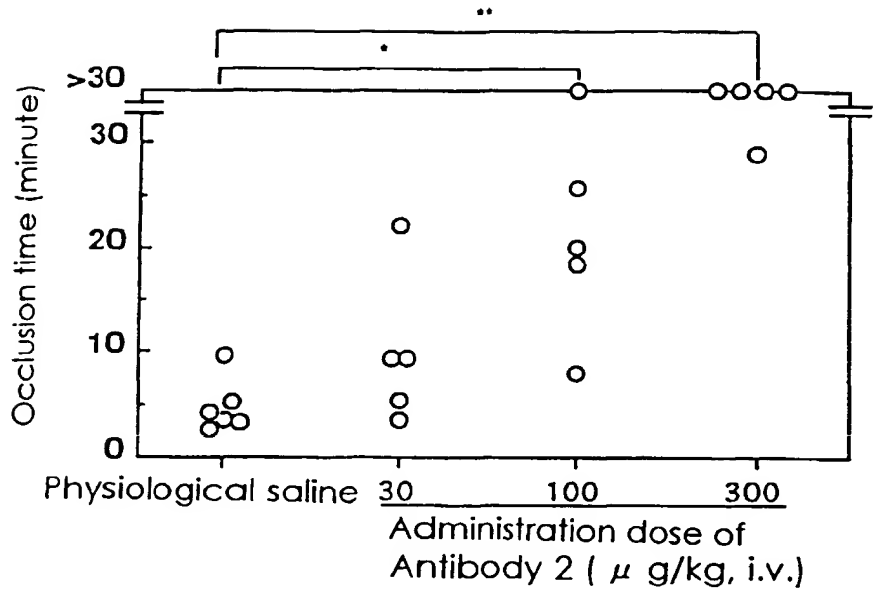
**
*
>30
30
20
10
0
Occlusion time (minute)
Physiological saline
30
100
300
Administration dose of
Antibody 2 (μ g/kg, i.v.)

FIG. 23

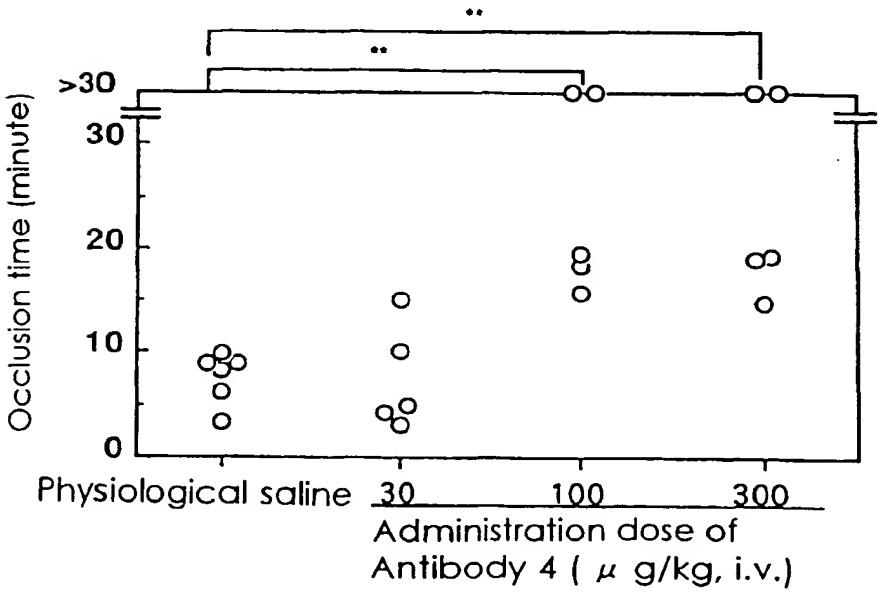
**
**
>30
30
20
10
0
Occlusion time (minute)
Physiological saline
30
100
300
Administration dose of
Antibody 4 (μ g/kg, i.v.)

FIG. 24

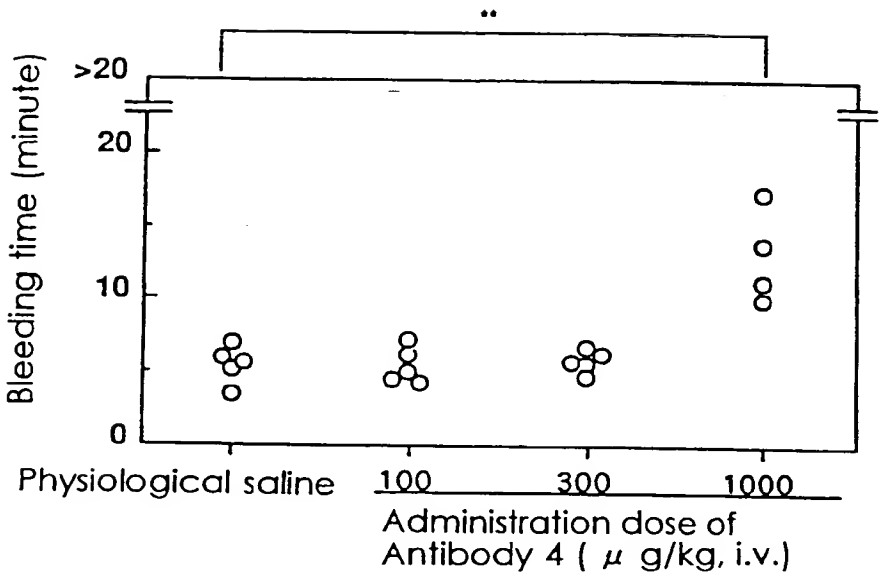
**
>20
20
10
0
Bleeding time (minute)
Physiological saline
100
300
1000
Administration dose of
Antibody 4 (μ g/kg, i.v.)

FIG. 27

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP95/02435

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Willebrand Factor Corresponding to the Platelet Glycoprotein IB Collagen and Heparin Binding Domains", Biochem, Biophys. Res. Commn., (1989) Vol. 164, No. 3, p. 1339-1347	
A	Aihara M et al. "Glycoprotein IB Has a Partial Role in Platelet-von Willebrand Factor Collagen Interaction", Thromb. Haemostasis, (1988), Vol. 60, No. 2, p. 182-187	1-9, 11-14 10
A	Jorieux et al. "Characterization of a Monoclonal Antibody to von Willebrand Factor as a Potent Inhibitor of Ristocetin-Mediated Platelet Interaction and Platelet Adhesion", Thromb. Haemostasis, (1987), Vol. 57, No. 3, p. 278-282	1-9, 11-14 10
A	Stel H V et al. "von Willebrand Factor in the Vessel Wall Mediates Platelet Adherence", Blood, (1985), Vol. 65, No. 1, p. 85-90	1-9, 11-14 10
A	Sixma J J et al. "Functional Domains on von Willebrand Factor Recognition of Discrete Tryptic Fragments by Monoclonal Antibodies That Inhibit Interaction of von Willebrand Factor with Platelets and with Collagen", J. Clin. Invest., (1984), Vol. 74, No. 3, p. 736-744	1-9, 11-14 10
A	Katzmann J A et al. "Mono Clonal Antibodies to von Willebrands Factor Reactivity with Porcine and Human Antigens", Blood, (1981), Vol. 58, No.3, p. 530-536	1-9, 11-14 10
A	Kobayashi S et al. "Intrahepatic Peribiliary Vascular Plexus in Various Hepatobiliary Deseases : a Histological Survey", Human Pathology, (1994) Vol. 25, No. 9, p. 940-946	1-9, 11-14 10
A	Chow T W et al. "Shear Stress-induced von Willebrand Factor Binding to Platelet Glycoprotein IB Initiates calcium Influx Associated with Aggregation", Blood, (1992), Vol. 80, No. 1, p. 113-120	1-9, 11-14 10

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